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Effects of Nd:YAG-Laser Irradiation on Monolayer Cell Cultures

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Background and Objective: **The clinical applications of Nd:YAG lasers on oral soft tissues include a wide field of surgical and periodontal procedures. This in vitro study focuses on the histological effects of Nd:YAG-laser irradiation on a fibroblast monolayer cell culture especially with regard to thermal damage and cell necrosis. The results of this basic research study provide us with clear power settings for a safe soft tissue laser treatment.**
Study Design/Materials and Methods: **Two hundred forty multiwell cell cultures and 24 micro-slide Leighton tubes were laser treated. Laser irradiation was performed with a commercial free-running pulse Nd:YAG laser and a quartz fiber with a diameter of 200 μm on L-929 fibroblast cell cultures. The variable parameters were pulse energy (30–120 mJ), pulse rate (20–100 Hz), power output (1.5–3.0 W), and time of irradiation (10–60 s). The cultures were analyzed with help of vital staining, autoradiography, and cytomorphology examination.**
Results: **Depending on the different settings the laser irradiation caused inhibitions of the DNA metabolism rate and the cell division rate, a degeneratively changed cytomorphology up to cell pyknosis. An increasing pulse energy, pulse rate, or an increased time of irradiation created an extended diameter of the pyknotic cell zone.**
Conclusions: **The laser beam creates an exactly bordered damage between cells. The cells had a very good inherent mobility, but the border between eliminated and unloaded cell zone was sharp, even after an incubation of 24 h. These stable results prove that the laser can be applied up to a micrometer distance. With the help of cell clusters it was proved that the laser beam is also able to eliminate exactly one monolayer. Cells which had been covered by another cell layer (in a cluster) were not eliminated. Lasers Surg. Med. 22:30–36, 1998.** **© 1998 Wiley-Liss, Inc.**

Key words: dentistry; Nd:YAG; laser; cell culture; temperature

INTRODUCTION

Neodymium:yttrium-aluminium-garnet lasers (Nd:YAG) are becoming more and more common in dental offices. The clinical applications of Nd:YAG lasers on oral soft tissues include a wide field of surgical and periodontal procedures [1–6].

However, even for small excisions, high-power settings and long irradiation times are necessary, resulting in an extensive zone of carbonization in collateral tissues [7]. This occurs because Nd:YAG laser irradiation is poorly absorbed in water, which represents the major component

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of biological tissues. So the laser irradiation penetrates the target tissue deeply, and high energy flow rates are necessary in order to heat up the tissue and to desiccate, carbonize, and vaporize the upper tissue layers [7].

According to Myers [8,9] pulse Nd:YAG lasers at low-power settings are as a matter of routine used for the treatment of periodontal pockets. But because of the penetration depth reaching the limbus alveolaris or even deeper regions, the thermal effect of the irradiation with infrared-lasers can cause a necrosis of the bone or in the periodontal membrane [10]. Myers claims that no negative effects can be registered if the power setting and pulse energy are conscientiously observed [8,9].

The thermal laser effects can be explained as following: Up to temperatures of 37°C, no pathological changes can be expected. Up to about 46°C, tissue damage is reversible. Around 60°C, the tissue and blood proteins denature (coagulate). At 100°C, the tissue water vaporizes. After the vaporization of the water, organic molecules are eliminated, resulting in a local tissue shrinkage by dehydration and cell contraction, carbonization, and vacuolization in the cell agglomeration. Finally, defects in the form of holes and cuts by tissue vaporization appear, and the creation of a zone of necrosis in the adjacent area can be found [11–16].

As a part of the basic research dealing with the effects of laser irradiation on oral soft tissues, this in vitro study focuses on the histological effects of Nd:YAG-laser irradiation on monolayer cell cultures under simple, standardized, and reproducible experimental conditions and determines the laser irradiation dose which selectively eliminates one of these monolayers.

The results of this study represent the basis for a corresponding in vivo study that is now in process in our clinic, which focuses on the penetration depth of the Nd:YAG laser beam during soft tissue applications.

MATERIALS AND METHODS

In this in vitro study 240 multiwell cell cultures and 24 micro-slide Leighton tubes were laser treated.

Based on a homogeneous cell suspension, the multiwell culture dishes (Falcon; Becton-Dickinson, Heidelberg, Germany), each dish containing 24 culture slides with a diameter of 12 mm and micro-slide Leighton tubes (Tecnormara, Fernwald, Germany), were bred with L-929-cell cultures (Strain 929, permanent cell-line; Gibco Limited Paisley, Scotland, Cat.No. 051-3004). The cultures were incubated in darkness for 24 h (one cell division) at +37°C. After the removal of the medium (Basal Medium Eagle [BME] with Earl's diploid salts [Gibco Limited, Paisley, Scotland, Cat.No. 073-01300T]), laser irradiation was performed from a distance of 2 mm with a commercial free-running pulse Nd:YAG laser (PulseMaster 1000™; Incisive Technologies, Inc., San Carlos, CA) and a quartz fiber with a diameter of 200 μm. The variable parameters were pulse energy (30 to 120 mJ), pulse rate (20–100 Hz), power output (1.5 to 3.0 W), and time of irradiation (10–60 s).

Vital Staining and Cytomorphology Examination

After laser irradiation, each culture was again supplied with 1 ml of the medium and then analyzed with help of a FDA/EB vital fluorescence staining (fluorescein diacetate/ethidium bromide vital fluorescence test and cytomorphology examination [haemalum staining with Mayer's Haemalum Solution; Merck, Darmstadt, Germany]).

Autoradiography

The DNA metabolism activity is a further valuation criteria. With the help of autoradiography (radioisotope: ^{3}H-thymidindesoxyribonucleotid = ^{3}H TdR, specific activity 40–60 Ci/mmol, Amersham/Buchler, Braunschweig, Germany, Cat. No. TRK 637 concentration in the BME 0.5 μCi/ml), different results in a single culture can be registered very clearly. Therefore medium and radioactive precursors were added to the culture after the laser irradiation and then incubated for 24 h. After the incubation the cultures were coated with a photo emulsion (Ilfort K5, Ilfort England, Ciba-Geigy Batch No. 27AK21/871) and exposed for 6 days in darkness for development. Cells which showed a metabolic activity were detected, whereas pyknotic cells did not incorporate the radioisotope.

Creation of Cell Clusters

In order to take off the cells, a mixture of trypsin, EDTA in BSS without $MgCl_2$ and $CaCl_2$ in distilled water was used. The trypsin has a dissolving effect on the outer parts of the cell membrane. After a longer duration of exposure some cells firmly cling together in form of so-called cell clusters and remain in this formation. In one part of our experiment cell cultures were

exposed to the enzyme mixture for 1 more minute in order to create these cell clusters.

The histological analysis was performed with help of a precision light microscope (Cambridge Instruments GmbH, Heidelberg, Germany). Outcome was assessed as follows:

1. Stimulation (increased cell growth compared to the control culture) [17]
2. Acceptance (same cell growth compared to the control culture) [17]
3. Inhibition (decreased cell growth compared to the control culture) [17]
4. Stagnation (no cell growth compared to the control culture) [17]
5. Toxicity (cell quantity decreased compared to the control culture) [17]

RESULTS

Tables 1 and 2 shows the employed laser irradiation parameters and resultant thermal effects. Table 1 depicts the laser damage at a fixed pulse energy while varying the other parameters, whereas Table 2 presents a fixed pulse rate while varying other parameters. A minus sign stands for unloaded cell cultures and a plus sign for thermally damaged cell cultures.

Thermal Effect

Both tables show that the experiment provides a sharp border between unloaded and thermally damaged cell cultures depending on the individual power settings. Table 1 proves that the occurrence of a thermal damage on the one hand depends on the increasing pulse energies, but on the other hand also on the time of irradiation. Some settings do not show any thermal damage for short irradiation times, but if the time of irradiation is increased, thermal damage occurs (for example: 30 mJ, 70 Hz, 2.1 W, 20 s: no thermal damage and 30 mJ, 70 Hz, 2.1 W, 30 s: thermal damage).

Table 3 shows that an increasing pulse rate and power output with a constant pulse energy and time of irradiation created an extended diameter of the pyknotic cell zone. The setting 30 Hz and 2,1 Watt created a diameter of 8,13 mm; at 100 Hz and 3.0 W a diameter of 10 mm was measured.

In order to show the different effects of the laser irradiation on the cellular level some characteristic results are reported.

Figure 1 shows (light microscope, ×90) a cell

TABLE 1. Laser Irradiation Parameters: Laser Damage After Treatment With a Fixed Pulse Energy and Varying Pulse Rate, Power Output, and Irradiation Duration

Pulse energy (mJ)	30									
Pulse rate (Hz)	50	70						80	90	100
Power output (W)	1.5	2.1						2.4	2.7	3.0
Time (s)	30	10	20	30	40	50	60	30	30	30
Thermal damage	–	–	–	+	+	+	+	+	+	+

Pulse energy (mJ)	40								60		80	
Pulse rate (Hz)	40	50	70						40	50	30	40
Power output (W)	1.6	2.0	2.8						2.4	3.0	2.4	3.2
Time (s)	30	30	10	20	30	40	50	60	30	30	30	30
Thermal damage	–	–	–	–	–	+	+	+	–	+	–	–

Pulse energy (mJ)	100							120					
Pulse rate (Hz)	20						30	20					
Power output (W)	2.0						3.0	2.4					
Time (s)	10	20	30	30	50	60	30	10	20	30	40	50	60
Thermal damage	–	–	+	+	+	+	+	+	+	+	+	+	+

TABLE 2. Laser Irradiation Parameters: Laser Damage After Treatment With a Fixed Pulse Rate and Varying Pulse Energy, Power Output, and Time Irradiation Duration

Pulse energy (mJ)	100						120						80	100	40	60	80	30	40	60							30						40						30	30
Pulse rate (Hz)	20												30		40			50									80												90	100
Power output (W)	2.0			2.4									2.4	3.0	1.6	2.4	3.2	1.5	2.0	3.0			2.1						2.8						2.4		2.7	3.0
Time (s)	30	10	20	30	40	50	60						30	30	30	30	30	30	30	30	10	20	30	40	50	60	10	20	30	40	50	60	30		30		30	30
Thermal damage	+	+	+	+	+	+	+						–	–	–	–	–	–	–	+	–	–	+	+	+	+	–	–	–	+	+	+	+		+		+	+

TABLE 3. Diameters of Thermal Damages After Treatment With Increasing Power Outputs

Pulse energy (mJ)	Pulse rate (Hz)	Power output (W)	Time (s)	Diameter of thermal damage (mm)
30	70	2.1	30	8, 13
30	80	2.4	30	9, 5
30	90	2.7	30	9, 33
30	100	3.0	30	10, 0

culture irradiated by Nd:YAG laser (setting: 30 mJ, 80 Hz, 2.4 W, 30 s) after vital staining.

On the left side of the figure vital cells in the thermally unloaded cell region are arranged like paving stones. Next to this region, in the border zone of the thermally damaged area, follows a diagonally arranged area of orange-stained cells. On the lower right side of the figure, the thermally damaged region begins where the vital staining was no longer successful. Because the fluorescence staining does not allow survey photographs, the minimum enlargement is ×90.

Figure 2 (light microscope, ×22.5) shows a cell culture irradiated by Nd:YAG laser with the same setting after haemalum staining. The region shown in the figure represents an area on the micro-slide of 4 × 6 mm. The laser beam creates an exactly bordered cell damage.

Figure 3 (light microscope, ×225) shows a cell culture irradiated by Nd:YAG laser with the above-mentioned setting after autoradiography. In the right region the damage zone is located, whereas the left half of the figure shows the well-marked unloaded cell region. The contrast between treated and untreated cells is very clear and sharp because of the blackening of the nuclei.

The cells in the unloaded region are arranged in a honeycomb fashion, without an orientation (lower left side). In the border area, the cells show an orientation toward the damage zone with wide cytoplasmic cell appendages. Here the migration of the undamaged cells into the damage zone starts. The fact that the border is still very sharp, even after an incubation of 24 h, leads to the conclusion that the margin of laser treatment can be applied exactly up to a micrometer distance.

After the laser treatment the cultures were incubated for 24 h. Although the cells have a very good inherent mobility, the border between damaged and unloaded cell zone is quite sharp. The cell damage is restricted to a circle with a diameter of 4 mm.

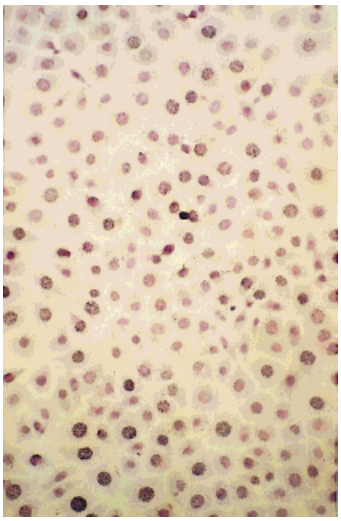

Fig. 6.

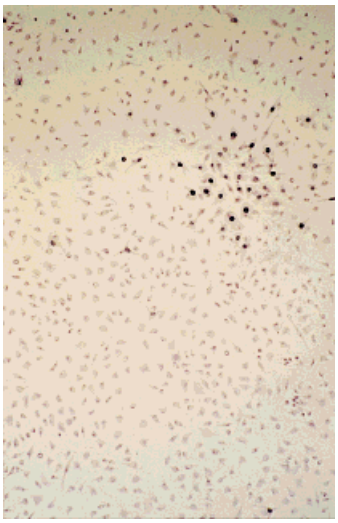

Fig. 7.

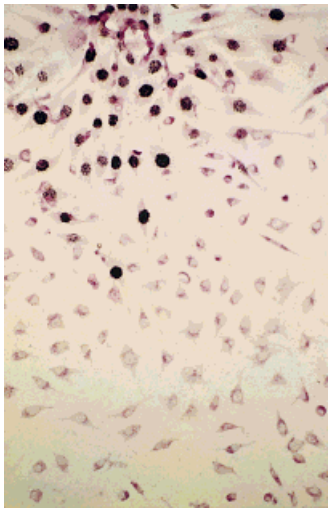

Fig. 8

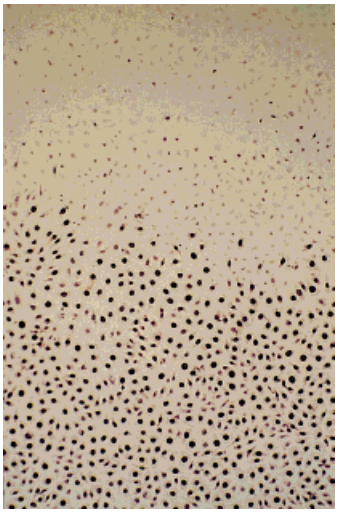

Fig. 3.

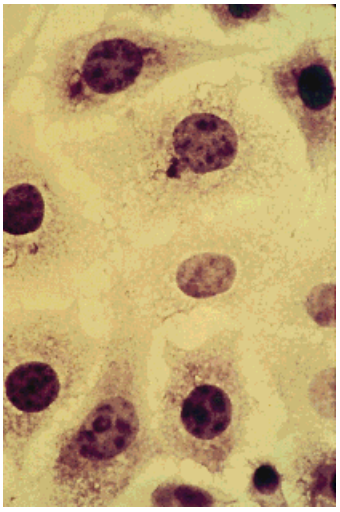

Fig. 4

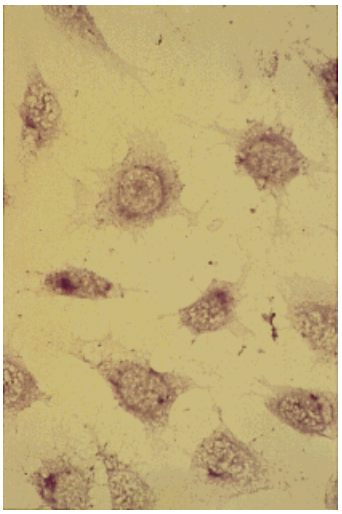

Fig. 5

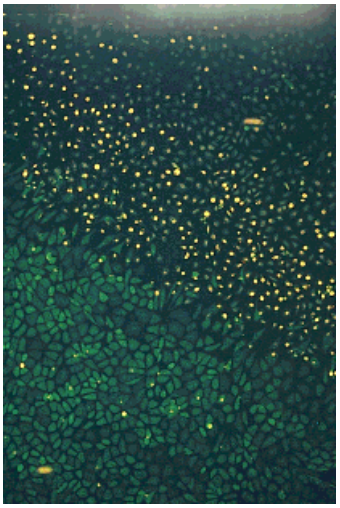

Fig. 1.

Fig. 2

Cytomorphology

A greater magnification (light microscope, ×900) of a culture irradiated by Nd:YAG laser with the above-mentioned setting clearly shows the morphological changes of the cytoplasmic and nuclear structure after haemalum staining (Figs. 4, 5). Both figures by light microscopy show 14 cells; the nuclear structure and the cell-spreading in the unloaded area is normal, and the staining is clear (Fig. 4). Figure 5 by light microscopy shows a more diffuse view, only little cytoplasm surrounds the nucleus which is difficult to identify. The staining and a well-structured nucleus with nucleoli is missing. The cytomorphology equals burned out cell corpses.

DNA Metabolism Activity

Figure 6 (light microscopy, ×225) shows a cell culture irradiated by Nd:YAG laser (setting: 100 mJ, 20 Hz, 2.0 W, 30 s) after autoradiography. The right part of the figure shows cells in the center of the thermal burdening, and the left part shows the border area of the unloaded culture. This setting causes the state of inhibition registered with the help of the DNA metabolism activity. The cells seem to be morphologically intact. The cell spreading is good and comes to 550 μm^2. Degenerative changes represented by vacuoles are not visible. The DNA metabolism activity is extremely reduced; about 70% of the cells show a staining but with clearly reduced staining intensity in comparison with Figure 5.

Fig. 1. Cell culture irradiated by Nd:YAG laser (setting: 30mJ, 80Hz, 2.4W, 30 seconds), vital staining ×90.

Fig. 2. Cell culture irradiated by Nd:YAG laser (setting: 30mJ, 80Hz, 2.4W, 30 seconds), haemalum staining ×225.

Fig. 3. Cell culture irradiated by Nd:YAG laser (setting: 30mJ, 80Hz, 2.4W, 30 seconds), autoradiography ×225.

Fig. 4. Cell culture irradiated by Nd:YAG laser (setting: 30mJ, 80Hz, 2.4W, 30 seconds), unloaded region, haemalum staining ×900.

Fig. 5. Cell culture irradiated by Nd:YAG laser (setting: 30mJ, 80Hz, 2.4W, 30 seconds), laser treated area, haemalum staining ×900.

Fig. 6. Cell culture irradiated by Nd:YAG laser (setting: 100mJ, 20Hz, 2.0W, 30 seconds), autoradiography ×225.

Fig. 7. Cell culture with a cell cluster irradiated by Nd:YAG laser (setting: 120mJ, 20Hz, 2.4W, 40 seconds), autoradiography ×90.

Fig. 8. Cell culture irradiated by Nd:YAG laser (setting: 120mJ, 20Hz, 2.4W, 40 seconds), cell cluster A, autoradiography ×2225.

Cell Clusters

The next figure by light microscopy shows a dose which exactly and irreversibly destroys a single monolayer. Figure 7 (light microscope, ×90) shows a cell culture irradiated by Nd:YAG laser (setting: 30 mJ, 70 Hz, 2.1 W, 30 s) after autoradiography with an experimentally created cell cluster.

The cell cluster visible in Figure 8 contained about 50 cells with a height of two cells. The height of the cell clusters was measured by turning a scaled micrometer screw of the precision light microscope. Having the first cell layer focused, the scale of the micrometer screw was calibrated. By turning the screw 15 degrees backward from the calibrating point the second cell layer was focused. By turning the screw more backward or even forward, no other cell layer could be focused. Through laser irradiation one cell layer was eliminated. In the following microscopical examination only one cell layer could be detected.

After the laser irradiation the cells in the center are still stratified, whereas the monolayer area is completely destroyed. So cells which are covered by another cell layer (in a cluster) survive and are still vital.

DISCUSSION

In this experimental set-up it was impossible to work in contact mode because of interactions between the laser and the multiwell dishes such as flash discharges or overheating of the dishes. So we decided to simplify and standardize this parameter as well as the other parameters, to allow a precise assessment of the results on the cellular level. Thus, we chose to work in non-contact mode with a distance of 2 mm between the glass fiber and the cell culture.

Our experiment shows that on the one hand the pulse energy but on the other hand also the time of irradiation represent a decisive parameter for the occurrence of a thermal damage. Especially the settings which can be regarded as a border between unloaded and thermally damaged cell cultures are of special interest.

After laser irradiation with 30 mJ, 70 Hz, 2.1 W for 20 s the analyzed cultures did not show any thermal damage, whereas an irradiation time of 30 s or more provides a thermally damaged cell culture.

The setting 100 mJ, 20 Hz, 2.0 W for 30 s, which also represents a "border setting" only created an inhibition of the DNA metabolism rate and the cell division rate, but not a degenatively changed cytomorphology. Although 70% of the cells apparently seem to be vital, as the staining shows, their metabolism activity is extremely reduced. This leads to the assumption that the laser beam has an effect on intracellular procedures without a obvious histological change of the cells' outward appearance.

The settings which do not belong to the group of "border settings" show a different result. The border between vital and pyknotic cells was much sharper after laser irradiation with 30 mJ, 80 Hz, 2.4 W for 30 s. This setting created an exactly bordered laser field with a diameter of 4 mm. The cells inside the affected area showed a spontaneous pyknosis.

The setting 120 mJ, 20 Hz, 2.4 W for 40 s caused a complete cell necrosis in a monolayer cell culture which could be showed by the examination of cell clusters. The striking result in this part of the experiment is that it is really possible to destroy only one single layer of cells without affecting the layer below. In connection with the very sharp border found with the following setting it can be claimed that the laser beam can be used extremely precisely, even with regard to the micrometer level.

The fact that an increasing pulse rate and output with constant pulse energy and time of irradiation created an extended diameter of the pyknotic cell zone leads to the conclusion that the length of irradiation also has an effect on the area of the cell necrosis. This shows that the necrotic area spreads out depending on the setting, although the diameter of the glass fiber is constant. This can be explained with the help of the thermal effects of laser beams mentioned above. After the complete vaporization of the tissue water the organic molecules are more destroyed, resulting in local tissue shrinking, carbonization, and vacuolization in the cell agglomeration. Finally, defects in the form of holes and cuts by tissue vaporization appear, and the creation of a zone of necrosis in the adjacent area can be found.

This basic results as a general idea of the effects of laser treatment on the cellular level help the dentist to understand the effects of his treatment, especially with regard to the expected wound healing, and represent the theoretical starting point of a corresponding in vivo study on patients in the section of gingivoplastic, gingivectomy, incisions, excisions, and laser-supported periodontal curettage performed in our clinic at the moment.

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REFERENCES

1. Wigdor HA, Walsh JT, Featherstone JDB, Visuri SR, Fried D, Waldvogel JL. Lasers in dentistry. Lasers Surg Med 1995; 16:103–133.
2. Midda M. Laser in periodontics. Periodontal Clin Invest 1992; 14:14.
3. Lin P, et al. A comparative effect of the Nd:YAG laser with root planing on subgingival anaerobes in periodontal pockets. J Dent Res 1992; 71:299, abstract 1547.
4. Landthaler M, et al. Nd:YAG laser therapy on oral verrucous leukoplakia. Dermatologica 1989; 178:115–117.
5. Leaderman RN, Feldman SJ, Cozean C. Anesthetic free periodontal surgery with a cw Nd:YAG laser. 4th International Congress on lasers in dentistry, Singapore 1994; abstract: 114.
6. White JM, Goodis HE, Rose CL. Use of the pulsed Nd:YAG laser for intraoral soft tissue surgery. Lasers Surg Med 1991; 11:455.
7. Keller U, Hibst R. Lasers in oral surgery. SPIE Vol. 2327 Medical Applications of Lasers II, 1994; 146–154.
8. Myers TD. "What lasers can do for dentistry and you." Dental Management 1989; 29:26–30.
9. Myers TD. Lasers in dentistry. J Am Dent Assoc 1991; 122:47.
10. Barahona RJF. Effect of irradiation with CO_2-laser on dog periodontal tissues. Bull Tokyo Med Dent Univ 1984; 31:165–186.
11. Berlien HP, Müller G. Angewandte Lasermedizin. Ecomed, Landberg/Lech 1989.
12. Grasser H, Ackermann K. Possibilities for the use of lasers in dental surgery. Dtsch Zahnarztl Z 1977; 32:512–515
13. Grotelüschen B. Laser in der operativen Medizin. Fortschr Med 1976; 94:33–36.
14. JGM Associates Inc. Dental applications of advanced lasers. May 1992.
15. Kato K, Nagasawa K, Nishikawa K. Thermal changes of soft tissue with irradiation of CO_2, Nd:YAG and argon laser. In: Atsumi K, Nimsakul N, eds. "Laser Tokyo '81 Inter Group Corp. Japan" 1981, pp. 17–22.
16. Weinberg W, Kromester A, Keiditsch E. Temperature. Measurement in Living Tissue Obtained in Laser Application. In: Kaplan I, ed. Laser Surgery, Jerusalem: Proc. 3rd Int. Congr. Acad. Press, 1979, vol 2, p 23.
17. Autian J, Dillingham EO. Overview of general toxicity testing with emphasis on special tissue culture tests. 1978; 21:70.